REMARKS

Upon entry of the present amendment, claims 1, 4-5, 11, 13-14, 20, 21 and 31 are pending in the present application. Applicants have amended claim 1 to more clearly define the genetically modified cells of the present invention. Claim 1, now dependent on claim 21, recites "natural antibody polypeptides" wherein the coding "polynucleotide" is operably linked to the promoter "in vitro." Basis for these amendments can be found in the Specification as originally filed, and in particular on pg. 4, lines 21–24 and pg. 5, lines 5–10. Applicants have further amended claim 1 to recite a polynucleotide element capable of secretion into "the blood circulation of a host mammal after the implantation of the mammalian non-plasmocyte cell into the host mammal." Basis for this amendment can be found in the Specification as originally filed, and in particular on pg. 4, lines 1–14. Applicants have also amended claim 1 to correct grammatical errors and antecedent basis. Claim 4 has been amended to more clearly define the cell according to the present invention. Specifically, claim 4 has been amended to recite a cell wherein the "nucleic acid is inserted in a vector." Basis for this amendment can be found in the Specification as originally filed, and in particular on pg. 6, lines 15–26. Claim 20 has been amended to recite a "natural antibody polypeptide." Basis for this amendment can be found in the Specification as originally filed, and in particular on pg. 5, lines 5–10. Claim 20 has also been amended to now recite the term "transfering upon transfection." Basis for this amendment can be found in the Specification as originally filed, and in particular on pg.6, line 6 through pg. 7, line 23, and pg. 13, lines 1–16.

The present amendment does not add new subject matter.

CLAIM OBJECTIONS

The Examiner has objected to claims 26, 27, 29, and 30, stating that the claims have various informalities.

Applicants have cancelled claims 26, 27, 29 and 30. Thus, Applicants assert that the present objections are now moot.

Accordingly, Applicants request withdrawal of the claim objections.

35 U.S.C. § 112, SECOND PARAGRAPH REJECTION

The Examiner has rejected claims 1, 3-8, 11-15, and 20-30 under 35 U.S.C. § 112, second paragraph, as allegedly being indefinite. Specifically, the Examiner has alleged that claims 1 and 20 are indefinite in their recitation of the claim terms "unmodified antibody polypeptide;" "a heavy chain;" "does not induce an immune response;" and "wherein the coding polypeptide is operably linked to..." The Examiner has also alleged that claim 1 is indefinite in its recitation of step (b) since it is unclear how method step (b) relates back to the preamble reciting "[t]he cell of claim 21" or whether the claim is in fact draw to a composition or a method of using said composition. Similarly, claim 20 is allegedly indefinite because it is unclear to the Examiner whether claim 20 is drawn to a method of making cells or a method of using cells. Claims 1 and 20 also recite the limitation "the coding polypeptide" in steps (iii) and (iv).

Applicants have cancelled claims 3, 6–8, 15 and 20. Applicants have amended claims 1 and 20 to more clearly claim and better define the present invention. Claim 1, now dependent on claim 21, recites "natural antibody polypeptides" wherein the coding "polynucleotide" is operably linked to the promoter "in vitro." Basis for these amendments can be found in the Specification as originally filed, and in particular on pg. 4, lines 21–24 and pg. 5, lines 5–10. Applicants have further amended claim 1 to recite a polynucleotide element capable of secretion into "the blood circulation of a host mammal after the implantation of the mammalian non-plasmocyte cell into the host mammal." Basis for this amendment can be found in the Specification as originally filed, and in particular on pg. 4, lines 1–14. Applicants have also amended claims 1 and 20 to correct grammatical errors and antecedent basis. Accordingly, Applicants believe that these amendments render the rejection moot.

The Examiner has also rejected claim 20 as indefinite in its recitation of the term "transferring."

Applicants have amended claim 20 to now recite the term "transfering upon transfection." Basis for this amendment can be found in the Specification as originally filed, and in particular

on pg.6, line 6 through pg. 7, line 23, and pg. 13, lines 1–16. Accordingly, Applicants believe that this amendment renders the rejection moot.

The Examiner has also rejected claims 21 and 31 as indefinite in their recitation of the term "operably linked."

Applicants have amended claims 21 and 31 to remove the recitation of the term "operably linked." Accordingly, Applicants believe that this amendment renders the rejection moot.

In light of the amendments above, Applicants respectfully request reconsideration and withdrawal of the present rejections.

35 U.S.C. § 112, FIRST PARAGRAPH REJECTION

The Examiner has rejected claims 1, 3-5, 11, 13-14, 20, 21 and 31 under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention. Specifically, the Examiner states:

> All of the instant claims recite "a nucleotide sequence element coding for a signal peptide operably linked to the nucleotide sequence coding for the antibody molecule fragment". However, there is no mention of signal peptides anywhere in the instant specification.

See December 14, 2000 Office Action at pg. 8.

Applicants disagree. Signal peptides are disclosed in Figure 1a and Figure 2a of the instant specification. These signal peptides correspond to the fragment comprising amino acids from position (-20) to position (-1) in the sequence presented in the figure 1a of the specification and the fragment comprising amino acids from position (-19) to position (-1) in the sequence presented in the figure 2a of the specification.

The fact that these sequences are signal peptides is documented in scientific literature, and assessed from information available in general databases as the Medline database from NCBI (www.ncbi:nlm.nih.gov:80/cntrcz/query.fcgi?db=Proteinou) or, a database such as IMGT related

to protein involved in immune system (http:/imgt/cnusc.fr:8104/). Cloning of a complete cDNA coding for a light chain or for a heavy chain of the Tg10 antibody to obtain secreting antibody cells clearly discloses, for those skilled in the art, that signal peptides are effectively present and consequently the DNA construction used to genetically modify cells comprise sequences coding for such signal peptides.

However, in order to expedite allowance of the present application, Applicants have amended claims 1, 20, 21 and 31 to remove the term "signal peptide."

Accordingly, Applicants respectfully request reconsideration and withdrawal of the present rejection.

35 U.S.C. § 112, FIRST PARAGRAPH REJECTION

The Examiner has rejected claims 1, 6, 20, and 31 under 35 U.S.C. § 112, first paragraph, as allegedly being non-enabled. The Examiner has stated:

> However, it is not clear in what way the sole ex vivo working example constitutes a "good demonstration" of anything other than perhaps an ability to secrete antibodies from implanted C2C12 cells in a syngeneic C3H [sic] mice, for purposes that are not entirely clear...The anti-Tgl0 working example merely demonstrates production of an antibody in the serum of syngeneic mice from implanted C2C12 cells. The specification, however, fails to provide a nexus between this type of implantation and the broad scope of implantations in humans, for example, using e.g. the broad scope of cells recited in claim 11.

See e.g., Office Action at pg. 11 through pg. 12, and pg. 14.

Applicants disagree. Applicants respectfully proffer that the Examiner's arguments regarding the correlation between murine and human enablement, and the supposed lack of ability of those of skill in the art to extrapolate from one cell type to another (when several different types have been proven to work well *in vitro and in vivo*), are without support. Also, Examiner's admission that "an ability to secrete antibodies from implanted C2C12 cells in . . . mice" is an important admission under the law and MPEP guidelines.

For example, the MPEP 2164.02 discusses working examples in a specification and the correlation between *in vitro* and *in vivo* data:

> The issue of "correlation" is related to the issue of the presence or absence of working examples. "Correlation" as used herein refers to the relationship between in vitro or in vivo animal model assays and a disclosed or a claimed method of use. An in vitro or in vivo animal model example in the specification, in effect, constitutes a "working example" if that example "correlates" with a disclosed or claimed method invention. If there is no correlation, then the examples do not constitute "working examples." In this regard, the issue of "correlation" is also dependent on the state of the prior art. In other words, if the art is such that a particular model is recognized as correlating to a specific condition, then it should be accepted as correlating unless the examiner has evidence that the model does not correlate. Even with such evidence, the examiner must weigh the evidence for and against correlation and decide whether one skilled in the art would accept the model as reasonably correlating to the condition. *In re Brana*, 51 F.3d 1560, 1566, 34 USPQ2d 1436, 1441 (Fed. Cir. 1995) (reversing the PTO decision based on finding that in vitro data did not support in vivo applications).

Since the initial burden is on the examiner to give reasons for the lack of enablement, the examiner must also give reasons for a conclusion of lack of correlation for an *in vitro* or *in vivo* animal model example. A rigorous or an invariable exact correlation is not required, as stated in *Cross v. Iizuka*, 753 F.2d 1040, 1050, 224 USPQ 739, 747 (Fed. Cir. 1985):

> [B]ased upon the relevant evidence as a whole, there is a reasonable correlation between the disclosed in vitro utility and an in vivo activity, and therefore a rigorous correlation is not necessary where the disclosure of pharmacological activity is reasonable based upon the probative evidence.

Applicants assert that the Examiner has supplied no objective evidence as to the alleged non-enablement, but simply offered a personal opinion. The present invention concerns antibody production and secretion that is made by a functional cells which do not normally produce nor secrete antibodies. It is known in the art that human muscle cells are able to secrete an ectopic protein after an appropriate genetic modification. For example, genetically modified cells have been injected into subjects in order to produce and secrete factor IX, and which are currently used to treat hemophilic patients. For example, see *Kay et al.*, Nature Genetics 24:257-261 (2000) (see attached). Also, *Baumgartner et al.*, Circulation 97:114-1123 (1998) (see attached), discusses the treatment of ischemic patients with genetically modified cells producing and secreting VEGF. Clinical trials with those modified cells (Factor IX or VEGF secreting cells) conducted in

humans were initially conducted in mice as a model animal for the preclinical trials. Hence, the mechanisms involved in secretion of a protein into the blood circulation of a mammal are very similar in different types of mammals and experiments conducted in mice can be extrapolated to humans for this kind of trial.

Furthermore, Applicants respectfully submit that the murine model is generally regarded within this field as the most appropriate model for studying cellular responses for testing new therapies for potential use in humans, and that the murine model is customarily used in this field to determine the potential enablement of such therapies in the treatment of humans. *See, e.g.,* Paul, *et al.,* eds., 1994. *Neoplastic Disease,* pp. 128-131 (Oxford Press); Zhang, *et al.*, 1994 *Cancer Gene Ther*, 1(1): 5-13 ("Zhang"); and Wills, *et al.*, 1994 *Hum Gene Ther*, 5: 1079-1088 ("Wills"). Zhang and Wills are directed toward the use of recombinant adenoviruses that express p53, a known tumor suppressor, in gene therapy of cancer. In both Zhang and Wills, the *in vivo* efficacy of the virus was first demonstrated using a murine model that is analogous to that used in the instant application. In particular, tumor cells were infected *ex vivo* and then injected into nude mice to "assess the ability of the recombinants to suppress tumor growth *in vivo*." (Wills, *et al.*) The results of these tests indicated that it was likely that p53 gene therapy would be useful as a treatment of existing tumors. This was supported by the demonstration of reduced tumor growth or prevention of tumor formation results, in Wills and Zhang, respectively. These references are, therefore, indicative of the fact that the murine model used in the present application is an art recognized method of testing gene therapies and is predictive of *in vivo* efficacy.

The murine model used in the present invention has been demonstrated to be predictive of the enablement of gene therapies in humans, as shown, for example, by Swisher, *et al.*, 1999 *J. Natl Cancer Inst*, 93(9): 763-771; Schuler, *et al.*, 1998 *Hum Gene Ther*, 9: 2075-2082; and Roth, *et al.*, 1996 *Nature Med*, 2(9): 985-991. These articles summarize the results of clinical trials initiated based on the results of preclinical studies, including those described in Zhang and Wills, which made use of this model as one of the preclinical validation tests (see above). As predicted by the results from the murine model tests, the administration of gene therapy vectors that express p53 were found to be therapeutically beneficial to many of the cancer patients tested.

Applicants, therefore, submit that the murine model used in the present application is an art recognized model that is predictive of enablememt of the present invention in other mammals, including humans.

As the art is such that a particular model is recognized as correlating to a specific condition, then it should be accepted as correlating. *See, Training Materials for Examining Patent Applications with Respect to 35 U.S.C. Section 112, first paragraph -- Enablement Chemical/Biotechnical Applications.* "Based upon the relevant evidence as a whole, there is a reasonable correlation between the disclosed *in vitro* utility and an *in vivo* activity, and therefore a rigorous correlation is not necessary where the disclosure of pharmacological activity is reasonable based upon the probative evidence." *Cross v. Iizuka*, 753 F.2d 1040, 1050, 224 USPQ 739, 747 (Fed. Cir. 1985).

The evidence provided by Applicant need not be *conclusive* but merely *convincing* to one skilled in the art. *Training Materials for Examining Patent Applications with Respect to 35 U.S.C. Section 112, first paragraph -- Enablement Chemical/Biotechnical Applications.* Thus, the Applicants, absent some *definitive* scientific basis to conclude that *in vivo* animal models do not correlate with other mammals in this instance, should be allowed to maintain the scope of their claims to all mammals, including humans, without providing clinical efficacy.

With respect to the requirement for human testing, the MPEP expressly states that human clinical data is *not* required for enablement under 35 U.S.C §112, first paragraph. For example, the MPEP states:

> Office personnel should not impose on applicants the unnecessary burden of providing evidence from human clinical trials. There is no decisional law that requires an applicant to provide data from human clinical trials to establish utility for an invention related to treatment of human disorders (*see, In re Isaacs*, 347 F.2d 889, 146 USPQ 193 (CCPA 1963); *In re Langer*, 503 F.2d 1380, 183 USPQ 288 (CCPA 1974)), even with respect to situations where no art-recognized animal models existed for the human disease encompassed by the claims.

See e.g., MPEP §2107.02.IV.

Additionally, the MPEP indicates that data from *in vitro* assays or testing in an animal model is generally sufficient:

> If reasonably correlated to the particular therapeutic or pharmacological utility, data generated using *in vitro* assays, or from testing in an animal model or a combination thereof almost invariably will be sufficient to establish therapeutic or pharmacological utility for a compound, composition or process.

See e.g., MPEP §2107.02.III. The MPEP further states that:

> The applicant does not have to prove that a correlation exists between a particular activity and an asserted therapeutic use of a compound as a matter of statistical certainty, nor does he or she have to provide actual evidence of success in treating humans where such a utility is asserted. Instead, as the courts have repeatedly held, all that is required is a reasonable correlation between the activity and the asserted use. *Nelson v. Bowler*, 626 F.2d 853, 857, 206 USPQ 881, 884 (CCPA 1980).

See e.g., MPEP §2107.02.I.

Regarding the Examiner's allegation that "undue experimentation" would be required by one skilled in the art to practice the present invention, Applicant respectfully reminds the Examiner that the amount of experimentation that is permissible to provide enablement depends upon a number of factors, which include: (1) the quantity of experimentation necessary, (2) the amount of direction or guidance presented, (3) the presence or absence of working examples, (4) the nature of the invention, (5) the state of the prior art, (6) the relative skill of those in the art, (7) the predictability of the art, and (8) the breadth of the claims. *In re Wands,* 858 F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988); MPEP §2164.01.

It is well established in patent law that a requirement of experimentation by a skilled person does not preclude enablement; all that is required is that the amount of experimentation not be *unduly extensive.* Additionally, "[t]he fact that experimentation may be complex. . . does not necessarily make it undue, if the art typically engages in such experimentation." *Massachusetts Institute of Technology v. AV Frotia,* 774 F.2d 1104, 227 USPQ 428 (Fed. Cir. 1985). "The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." *In re Wands*, 858 F.2d at 737, 8 USPQ2d at 1404 (*citing, In re Angstadt*, 537 F.2d 489, 502-04,

190 USPQ 214, 218 (CCPA 1976)). Similarly, "[t]ime and difficulty of experiments are not determinative if they are merely routine." *United States v. Telectonics Inc.*, 857 F.2d 778, 8 USPQ2d 1217 (Fed. Cir. 1988), *cert. denied*, 490 U.S. 1046 (1989).

As previously discussed the present invention provides a great deal of support for the production and secretion of antibodies from the claimed cells disclosed herein. Any additional experimentation which may be subsequently required in the practice of the invention would merely entail following the methods disclosed within the Specification which comprise *conventional* assays and guidelines *known* within the art. This type of well-supported, well-known, and clearly delineated experimentation is allowed under 35 U.S.C §112, first paragraph.

Accordingly, Applicants respectfully request reconsideration and withdrawal of the present rejection.

35 U.S.C. § 102(B) REJECTION (WRIGHT ET AL.)

Claims 4 and 13 stand rejected under 35 U.S.C. § 102(b) as allegedly being anticipated by Wright *et al.*, (Crit. Rev. Immunol., 12(3,4):125-168, 1992) (*Wright*). Applicants respectfully traverse. Anticipation requires the disclosure in a single prior art reference of each element of the claim under consideration. "There must be no difference between the claimed invention and the reference disclosure, as viewed by a person of ordinary skill in the field of the invention." *Scripps Clinic & Research Foundation v. Genentech Inc.*, 18 USPQ 2d 1001, 1010 (Fed. Cir. 1991).

Wright does not disclose the claimed invention. *Wright* concerns the production of monoclonal antibodies *in vitro* by non-B cell lines, with the purpose of purifying the monoclonal antibodies from the culture supernatants. *Wright* does not disclose a method for the production of monoclonal antibodies *in vivo*. The Applicant's approach, which is clearly described in the present Specification, page 5 lines 15-18, and claimed as independent claim 21, describes monoclonal antibodies that are produced by genetically modified non-B cells which are implanted into a host mammal and then secrete the monoclonal antibodies into the host mammal. Consequently, the dependant claims 4 and 13 are not anticipated by *Wright*.

Accordingly, Applicants respectfully request reconsideration and withdrawal of the present rejection.

35 U.S.C. § 102(B) REJECTION (STEVENSON ET AL.)

Claims 4, 11-13, and 21 stand rejected under 35 U.S.C. 102(b) as allegedly being anticipated by Stevenson *et al.*, (Ann. N.Y. Acad. Sci., 772:212-226, 1995) (*Stevenson*). Applicants respectfully traverse.

The disclosure of *Stevenson* concerns only the teaching of chimeric antibodies constructed with nucleic acid fragments. *Stevenson* does not disclose the release of a natural antibody by genetically modified cells into the blood circulation of a mammal. Since claims directed to antibody fragments have been canceled and amended from the present application, *Stevenson* does not anticipate the present claims. Furthermore, *Stevenson* discloses a vaccine approach involving injection of a **vector** into a body, while the present application is based on direct antibody secretion by **implanted** cells. Thus, *Stevenson* does not anticipate the present invention.

Accordingly, Applicants respectfully request reconsideration and withdrawal of the present rejection.

35 U.S.C. § 102(B) REJECTION (MORITZ ET AL.)

Claims 4-5, 11, 13 and 21 stand rejected under 35 U.S.C. 102(b) as allegedly being anticipated by Moritz *et al.*, (Proc. Natl. Acad. Sci. USA, 91:43 18-4322, 1994) (*Moritz*). Specifically, the Examiner alleges that *Moritz* discloses cells not naturally producing antibodies (*e.g.* cytotoxic T-lymphocytes) containing a viral vector (retroviral) capable of expressing an antibody in the blood circulation of mammals.

Applicants traverse. *Moritz* does not anticipate the pending claims. *Moritz* does not introduce the recombinant cells into a mammal, as it merely discloses chimeric antibody constructed with scFv nucleic acid fragments. Since claims directed to fragment antibody has

been canceled and this publication does not disclose the secretion of natural antibodies in the blood circulation of mammals, *Moritz* does not anticipate the above mentioned claims.

Accordingly, Applicants respectfully request reconsideration and withdrawal of the present rejection.

35 U.S.C. § 102(B) REJECTION (CHEN ET AL. (1994))

Claims 4, 11, 14 and 21 stand rejected under 35 U.S.C. 102(b) as allegedly being anticipated by Chen *et al.*, (Proc. Natl. Acad. Sci. USA, 91:5932-5936, 1994) (*Chen I*). Specifically, the Examiner alleges that the arguments set forth in the response are not persuasive since primarily because they rely on features (i.e., implantation of cells, antibodies reaching the blood circulation, etc.) that are not recited in the rejected claim(s)." *See e.g.*, Office Action at pg. 20.

Applicants respectfully traverse. *Chen I* does not anticipate the pending claims. *Chen I* discloses mammalian non-plasmocyte cells that are useful only for the secretion of Fab **fragment antibodies** into the blood circulation of mammals. *Chen I* does not teach the secretion of a **natural antibodies** into the blood circulation of mammals.

However, in order to expedite allowance of the present application, Applicants have amended independent claim 21 (and therefore dependant claims 4, 11, and 14) to recite the features disclosed by the specification such as the implantation of cells, and secretion of antibodies into the blood circulation of a mammal. Thus, *Chen I* does not anticipate the present claims.

Accordingly, Applicants respectfully request reconsideration and withdrawal of the present rejection.

35 U.S.C. § 102(B) REJECTION (CHEN ET AL. (1996))

Claims 4-5, 11, 14, and 21 stand rejected under 35 U.S.C. 102(b) as allegedly being anticipated by Chen *et al.*, (Hum. Gene Ther., 7:1515-1525, 8/1996) (*Chen II*). Applicants respectfully disagree. *Chen II* discloses mammalian non-plasmocyte cells that are useful only for

the secretion of Fab **fragment antibodies** into the blood circulation of mammals. *Chen II* does not teach the secretion of a **natural antibodies** into the blood circulation of mammals. Since claims directed to antibody fragments have been canceled and amended in the present application, *Chen II* does not anticipate the present claims.

CONCLUSION

On the basis of the foregoing amendments and remarks, Applicants respectfully submit that the pending claims are in condition for allowance. If there are any questions regarding these amendments and remarks, the Examiner is encouraged to contact either of the undersigned at the telephone number provided below.

Respectfully submitted,

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VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the Claims:

Please cancel claims 3, 6, 12 and 22-30 without prejudice, and amend claims 1, 4, 20, 21 and 31 as follows:

1. (Twice Amended) The cell of claim 21,

(a) wherein said nucleic acid comprises a polynucleotide coding for a natural [an unmodified] antibody polypeptide,

[(i) wherein the unmodified antibody polypeptide is a heavy chain or function fragment thereof:

(ii) wherein the antibody polypeptide does not include an immune response neutralizing the antibody polypeptide;

(iii)] (i) wherein the coding polynucleotide [polypeptide] is operably linked to a promoter in vitro for expressing the polynucleotide encoding the antibody polypeptide in the mammalian non-plasmocyte cell in vivo; and

[(iv)] (ii) wherein the coding polynucleotide [polypeptide] is operably linked to a polynucleotide element coding for a signal peptide required for the secretion of the antibody polypeptide from the mammalian non-plasmocyte cell into the blood circulation of a host mammal after the implantation of the mammalian non-plasmocyte cell into [a] the host mammal. [genetically modified by said nucleic acid sequence and not naturally producing antibodies; and

(b) wherein the mammalian non-plasmocyte cell comprising the nucleic acid has been introduced into the host mammal.]

4. (Twice Amended) The cell of claim 21, wherein the nucleic acid is inserted in a vector.

20. (Twice Amended) A method of making a mammalian non-plasmocyte cell comprising a nucleic acid containing a polynucleotide coding for a natural [an unmodified] antibody polypeptide, comprising the steps of:

(1) transfering upon transfection at least one nucleic acid comprising a polynucleotide coding for said natural [an unmodified] antibody polypeptide,

[(i) wherein the unmodified antibody polypeptide is a heavy chain or functional fragment thereof;

(ii) wherein the antibody polypeptide does not induce an immune response sufficient for neutralizing the antibody polypeptide;

(iii)] (i) wherein the coding polynucleotide peptide is operably linked to a promoter for expressing the polynucleotide encoding the antibody polypeptide in the mammalian non-plasmocyte cell; and

[(iv)] (ii) wherein the coding polynucleotide is operably linked to a polynucleotide element coding for a signal peptide required for the secretion of the antibody polypeptide from the mammalian non-plasmocyte cell into the blood circulation of a host mammal after the implantation of the mammalian non-plasmocyte cell [;

(2) culturing the cell in vitro; and

(3) implanting [introducing] the cell into a host mammal.]

21. (Amended) A mammalian non-plasmocyte cell genetically modified with a nucleic acid, wherein the nucleic acid comprises a nucleotide sequence coding for an antibody molecule:

(a) wherein the nucleotide sequence coding for the antibody molecule is operably linked to a promoter for expressing said nucleotide sequence encoding the antibody molecule in the mammalian non-plasmocyte cell, and

(b) wherein the nucleic acid comprises a sequence for termination of the transcription, situated downstream from the sequence coding for an antibody molecule and permitting [a nucleotide sequence element coding for a signal peptide operably linked to the nucleotide sequence coding for the antibody molecule, for] the secretion[ng] of said antibody molecule from the mammalian non-plasmocyte cell into the blood circulation of a host mammal after the implantation of the mammalian non-plasmocyte cell into [a] the host mammal.

31. (Amended) A method for delivering an antibody to the blood system of a host mammal, comprising: implanting a cell into a mammal,

(a) wherein the implanted cell is a mammalian non-plasmocyte cell genetically modified with a nucleic acid, wherein the nucleic acid comprises a nucleotide sequence coding for an antibody molecule;

(b) wherein the nucleotide sequence coding for the antibody molecule is operably linked to a promoter for expressing said nucleotide sequence coding the antibody molecule in the mammalian non-plasmocyte cell; and

(c) wherein the nucleic acid comprises a sequence for termination of the transcription, situated downstream from the sequence coding for an antibody molecule and permitting [a nucleotide sequence element coding for a signal peptide operably linked to the nucleotide sequence coding for the antibody molecule, for] the secretion[ng] of said antibody molecule from the mammalian non-plasmocyte cell into the blood circulation of a host mammal after the implantation of the mammalian non-plasmocyte cell into [a] the host mammal.

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Axin' cancer

The mouse that *Rored*

Substituting chromosomes

Waiting to inhale

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Evidence for gene transfer and expression of factor IX in haemophilia B patients treated with an AAV vector

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Pre-clinical studies in mice and haemophilic dogs have shown that introduction of an adeno-associated viral (AAV) vector encoding blood coagulation factor IX (F.IX) into skeletal muscle results in sustained expression of F.IX at levels sufficient to correct the haemophilic phenotype[1,2]. On the basis of these data and additional pre-clinical studies demonstrating an absence of vector-related toxicity, we initiated a clinical study of intramuscular injection of an AAV vector expressing human F.IX in adults with severe haemophilia B. The study has a dose-escalation design, and all patients have now been enrolled in the initial dose cohort (2×10^{11} vg/kg). Assessment in the first three patients of safety and gene transfer and expression show no evidence of germline transmission of vector sequences or formation of inhibitory antibodies against F.IX. We found that the vector sequences are present in muscle by PCR and Southern-blot analyses of muscle biopsies and we demonstrated expression of F.IX by immunohistochemistry. We observed modest changes in clinical endpoints including circulating levels of F.IX and frequency of F.IX protein infusion. The evidence of gene expression at low doses of vector suggests that dose calculations based on animal data may have overestimated the amount of vector required to achieve therapeutic levels in humans, and that the approach offers the possibility of converting severe haemophilia B to a milder form of the disease.

Haemophilia B is the bleeding diathesis resulting from mutations in the gene encoding F.IX (*F9*), a proenzyme required for generation of a fibrin clot. The clinical severity of haemophilia B correlates closely with circulating levels of F.IX: individuals with less than 1% of normal activity are severely affected, whereas those with levels 1–5% of normal generally have a more moderate course. Current treatment is based on intravenous infusion of clotting factor concentrates; regimens in which factor is infused prophylactically, with a goal of maintaining factor levels greater than 1% at all times, have been shown to prevent most of the joint damage and life-threatening bleeding complications of the disease[3,4]. Thus, the goal of gene therapy for haemophilia B is the sustained expression of F.IX at levels more than 1% of normal. This goal has been achieved in mice and haemophilic dogs by introducing an AAV vector expressing F.IX into skeletal muscle. Intramuscular injection of an AAV vector expressing human F.IX into immunodeficient mice caused expression of F.IX at 5–7% of normal human plasma levels for more than 12 months (vector dose of 1×10^{13} vector genomes (vg)/kg; ref. 1). Subsequently, intramuscular injection of an AAV vector expressing canine F.IX in dogs with haemophilia B resulted in expression of F.IX at levels up to 1.4% of normal (vector dose of 8.5×10^{12} vg/kg; ref. 2). The levels of expression in these haemophilic dogs are currently stable more than 2.5

Table 1 • Clinical data for patients A, B and C

	A	B	C
Age	38	23	67
Race	European	Asian	European
Baseline F.IX activity level	<1%	<1%	<1%
Baseline F.IX antigen level	24%	<1%	<1%
Mutation	Arg-4→Leu nt 6,365 CGG→CTG	Ala 351→Pro nt 31,172 GCT→CCT	Gly 114→Arg nt 17,755 GGA→CGA
Viral status			
HIV	positive	negative	negative
hepatitis C	positive	positive	negative
hepatitis B	negative	negative	positive
hepatitis A	negative	negative	negative
Significant medical history	s/p GI bleed; s/p seizures secondary to bilateral epidural haematomas; s/p eosiniphilic granuloma R parietal skull; s/p knee synovectomy and arthroscopy	s/p GI bleed; s/p nephrectomy secondary to iliopsoas bleed mellitus	s/p GI bleed; adult onset diabetes
Current medications	ritonavir, lamivudine, stavudine, oxycodone prn	oxycodone prn	glyburide

Departments of [1]Pediatrics, [2]Genetics and [3]Surgery, Stanford University School of Medicine, Palo Alto, California, USA. [4]The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania, USA. Departments of [5]Pediatrics and [6]Surgery, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA. [7]Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania, USA. [8]Avigen, Inc., Alameda, California, USA. Correspondence should be addressed to K.A.H. (e-mail: high@email.chop.edu).

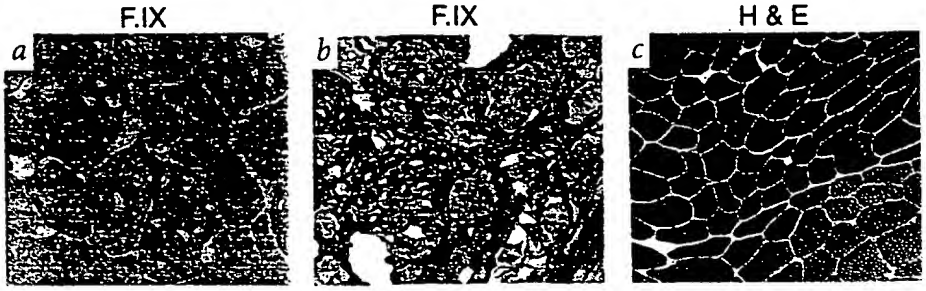


Fig. 1 Histochemical analysis of muscle biopsy. Immunoperoxidase staining of F.IX is shown for cross-sections of muscle tissue of a negative control (*a*) and a vector-injected patient (*b*). The dark brown staining for F.IX is seen in the extracellular matrix surrounding muscle fibres. Original magnification ×200. *c*, Haematoxylin-and-eosin–stained cross-section of muscle tissue from a patient injected with vector. Original magnification ×100. Muscle biopsies were performed 2–3 months after vector administration.

years after the initial and only injection (R.W.H., K.A.H. and T. Nichols, unpublished data).

Clinical data on our first three patients are shown (Table 1). Evidence for gene transfer and expression following vector administration was sought directly by muscle biopsy and indirectly by measurement of circulating F.IX levels and assessment of bleeding episodes and frequency of clotting factor infusion. We performed muscle biopsies 8–12 weeks after vector administration; PCR on DNA extracted from injected muscle was positive for vector sequences in all three patients (data not shown). Immunohistochemical staining of skeletal muscle was positive for F.IX in the extracellular space, a pattern that had been documented in pre-clinical studies for F.IX secreted by muscle fibres[1] (Fig. 1*a*,*b*). Additional sections analysed by routine histology showed no evidence of inflammation or muscle injury (Fig. 1*c*, and data not shown). Results of coagulation assays and records of factor usage for patients A and B are shown (Fig. 2 and Table 2). Patient A, who was documented to have a baseline F.IX level of less than 1% by three clinical coagulation laboratories, demonstrated a level of more than 1% (also documented by three clinical coagulation laboratories) on multiple occasions beginning approximately 8 weeks after vector administration. These levels were drawn at time points at least 14 days after the most recent factor infusion, eliminating the possibility that the levels reflected a contribution from residual infused factor. Patient B showed a small change in F.IX level, remaining less than 1% of normal (Table 2), but both patients showed a reduction in clotting factor consumption following treatment with the AAV vector (Fig. 2). The treatment time lines are given in 20-day intervals; the first half of the time line (pre-AAV treatment) serves as a control for the second half (post-AAV treatment). Patient A has experienced a 50% reduction in factor usage sustained over a period of more than 100 days, and patient B has experienced an 80% reduction in factor usage also sustained over a period of more than 100 days. Patient C, despite a F.IX level of less than 1%, treats himself infrequently (so-called 'mild-severe'[5]), typically less than four times per year. Since vector injection five months ago, he has had no change in clinical status or F.IX levels (data not shown). Gene transfer and expression, however, were documented by Southern blot on DNA extracted from a muscle biopsy specimen, which showed approximately one vector genome copy per diploid genome, and by RT–PCR, which was positive for F.IX expression (data not shown).

Major safety issues to be addressed here include the risk of formation of inhibitory antibodies to the transgene product, which can block treatment by conventional protein therapy, and the risk of inadvertent germline transmission of vector sequences. Evidence for formation of anti-F.IX antibodies was sought by two different methods, the standard Bethesda assay and a western-blot method. Bethesda assay performed weekly through the first eight weeks, then biweekly through the next four months, showed no evidence of inhibitor formation (data not shown). Western-blot analysis, which detects both inhibitory and non-inhibitory antibodies, has also been consistently negative for evidence of antibodies (Fig. 3*a*). The clinical responses of the patients to infused factor, and a pharmacokinetic study completed in one patient (data not shown), support these laboratory studies, because all patients continue to exhibit excellent responses to clotting factor concentrates.

Pre-clinical biodistribution studies in mice and rabbits carried out at doses 50-fold higher than those used here demonstrated that AAV vector introduced into sites in skeletal muscle remains largely confined to that tissue. Specifically, there is no evidence of distribution of vector into the semen (ORDA web site, http://www.nih.gov/od/orda/3-99RAC.htm) despite transient low-level positive signals in serum 24 hours after injection.

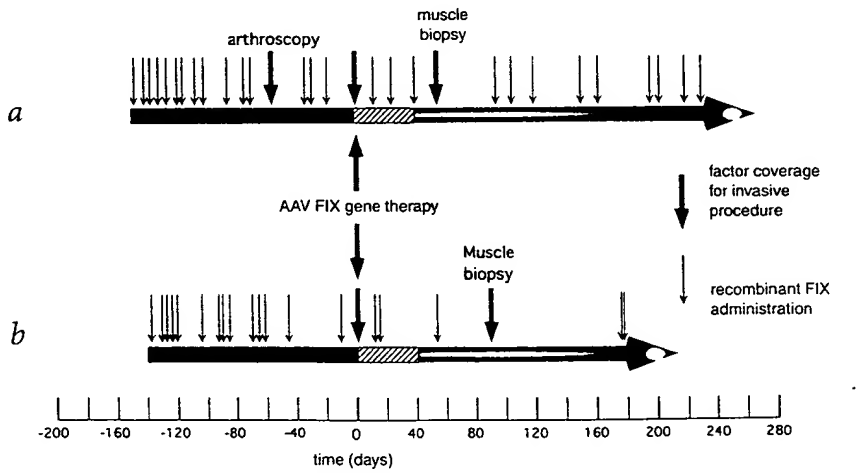


Fig. 2 Factor usage for patients A and B. The horizontal line denotes time; the scale at the bottom is marked in 20-day increments. Arrows denote infusion of F.IX concentrate for spontaneous bleeds (thin arrows) or invasive procedures (thick arrows). The thick vertical arrow in the middle of the chart denotes the date of vector infusion. The hatched bar on the timeline denotes the initial six-week period during which transgene expression is expected to be low based on animal studies[2]. All patients have baseline F.IX levels <1%. ***a***, For patient A, F.IX was documented to be 1%, with an activated partial thromboplastin time (aPTT) of 61 s, when he presented for muscle biopsy at 8 weeks following injection. On the day of and 1 d after muscle biopsy, the patient received F.IX concentrate; after 17 d, with no intervening factor treatment, the F.IX level was 1.6% with an aPTT of 48 s. Ten days later, the F.IX level was determined to be 1.4% with an aPTT of 47 s, again with no intervening factor treatment. Ten days later the patient treated himself with concentrate for atypical knee pain, and a F.IX level drawn after 4 d was 3.7% with an aPTT of 41 s, reflecting the recent protein infusion. A blood sample drawn 14 d after a subsequent treatment showed a F.IX level of 1.3% with an aPTT of 50 s. Over the ensuing weeks the factor level was measured in the 0.5–1.0% range, with aPTTs in the range of 50 s. Factor infusion is reduced ~50% from baseline. ***b***, The baseline F.IX level of patient B is <0.3%; his baseline factor infusion is ~2–5 times/month. Despite no substantial change in F.IX level, patient B's factor consumption has decreased by >80%.

Table 2 • Coagulation data[a] for patients A and B

	Patient A[b]			Patient B[bc]		
	F.IX	aPTT		F.IX	aPTT	
Baseline	<1%			<0.3%		
Week 6	<0.3%	82.9				
Week 8	1%	61		<0.3%	102	
Week 10	1.6%	48		0.3%	91.2	
Week 12	1.4%	46.8		0.3%	102.3	
Week 14	3.7%	41.0	(post-F.IX infusion)	3.0%	52.6	(post-F.IX infusion)
Week 17	1.3%	50.6		0.4%	72	
Week 18	0.8%	49.4				
Week 20	0.5%	54.1		0.4%	107	
Week 22	0.9%	53.7				
Week 24	0.5%	52.1		0.8%	65.5	

[a]Unless otherwise noted, all data points were drawn at least 14 d after the most recent factor transfusion. [b]Data generated in CHOP Clinical Coagulation Laboratory. [c]Data generated in Stanford University Clinical Coagulation Laboratory.

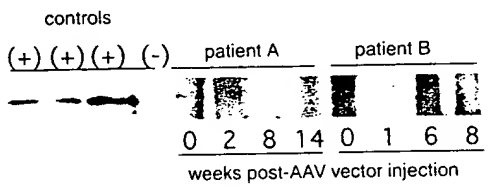
PCR analysis for vector sequences in body fluids from patients (data not shown) is in agreement with the pre-clinical studies, as serum samples were positive for vector sequences at 24 and 48 hours after injection, but were negative thereafter. Saliva samples were also positive at 24 hours after injection, but were subsequently negative, and one patient had a positive urine sample at 24 hours with all subsequent urine samples being negative. All remaining samples, including serial semen samples collected out to 48 days, 56 days and 59 days after injection, were negative for vector sequences.

The effect on transduction efficiency in skeletal muscle of neutralizing antibodies against AAV serotype 2 is unknown[6]. All patients enrolled in this study had detectable titres of neutralizing antibodies against AAV before treatment, with the titre varying over a range of two logs, from 1:10 to 1:1,000 (Fig. 3*b*). The rise in neutralizing antibody titre following vector administration varied from 10- to 1,000-fold. The highest pre-treatment antibody titre was in patient B, whose post-injection muscle biopsy is positive for F.IX expression by immunohistochemical staining (Fig. 1*b*), arguing against any inhibitory effect of the antibodies on skeletal muscle transduction. Additional laboratory studies, including serial complete blood counts and serum chemistries, disclosed no treatment-related abnormalities (data not shown).

Despite promising pre-clinical data, clinical experience with AAV vectors is limited; our study is the first in which AAV vectors have been introduced into skeletal muscle. On the basis of these initial patients, the approach appears to be safe, with no evidence at this dose for toxicity related to vector administration, inadvertent germline transmission of vector sequences or formation of inhibitory antibodies to the transgene product. Moreover, biopsy of injected sites shows evidence of gene expression by immunofluorescence staining. Notably, one of the patients in the initial low-dose cohort showed detectable circulating levels of F.IX above 1%. On the basis of studies in mice and haemophilic dogs[1,2], we had predicted that the patients in the low-dose group would not show measurable levels of F.IX expression (Table 3). Our observations suggest that the vector may be more efficient in humans than in mice or dogs; indeed, we have observed this to be the case in tissue culture, where we have measured as much as a 2-log difference in copy number of the donated gene in primary cultures of human versus mouse muscle cells (unpublished data). Because the vector is engineered from a virus that infects humans but not rodents, the processes of vector binding and entry[7,8] may be more efficient in human cells than in those of other species. A similar consideration applies to the CMV promoter-enhancer used in the vector; because CMV infects humans but not other species, the promoter may have evolved to express most efficiently in the setting of human transcription factors. An objective of dose escalation will be to identify a dose at which all patients express F.IX levels of more than 1%.

The fact that F.IX levels of just above 1% in patient A were associated with a reduction in factor use is consistent with the findings of the Swedish prophylaxis studies, which showed a reduction in haemorrhages when concentrate was dosed to maintain nadir levels of approximately 1% (refs 3,4). The reduction in bleeding seen in patient B raises the question of whether levels of F.IX less than 1% can also result in a reduction in clinical bleeding episodes. More data will be required to resolve this issue. The difference in factor levels seen among patients A, B and C may be accounted for by biologic variation, but another factor that may be important is the presence or absence of circulating F.IX antigen (Table 1). The volume of distribution of F.IX includes both the intravascular and extravascular space, where F.IX can bind tightly to collagen IV (ref. 9). Saturation of these potential binding sites in individuals with circulating F.IX protein may result in higher levels of the donated gene product in the

a



b

time post vector injection	patient A	patient B	patient C
pre-treatment	1:100	1:1,000	1:10
1 week	1:1,000	1:10,000	1:1,000
2 weeks	1:5,000	1:10,000	1:10,000
4 weeks	NS	1:10,000	1:10,000
6 weeks	NS	1:10,000	NS
8 weeks	1:5,000	1:10,000	NS
12 weeks	1:1,000	NS	NS

Fig. 3 Immune responses to AAV-CMV-hF.IX. ***a***, Western-blot analysis of anti-human F.IX in serum samples of haemophilia B patients. Plasma-derived human F.IX is transferred to a membrane, which is incubated with serum samples from patients. Lanes 1,2, positive control (+) (patient with inhibitory anti-F.IX) diluted 1:2,000; lane 3, positive control (+) diluted 1:1,000; lane 4, negative control (–); lanes 5–8, samples from patient A pretreatment (0 weeks, lane 5) and 2 weeks (lane 6), 8 weeks (lane 7) and 14 weeks (lane 8) following AAV-vector injection; lanes 9–12, samples from patient B pretreatment (0 weeks, lane 9) and 1 week (lane 10), 6 weeks (lane 11) and 8 weeks (lane 12) post-injection. ***b***, Neutralizing antibody titres against AAV before and after treatment with AAV-CMV-hF.IX. NS, no sample available at time of assay.

Table 3 • Predicted levels of circulating F.IX in humans

Dose	F.IX level in mice[a]	F.IX level in dogs[b]	Predicted level in humans[c]	Predicted % normal levels in humans
2×10^{11} vg/kg	6 ng/ml	2–4 ng/ml	2–6 ng/ml	≤0.1%
2×10^{12} vg/kg	60 ng/ml	16 ng/ml	16–60 ng/ml	0.3–1.2%
1×10^{13} vg/kg	300 ng/ml	80 ng/ml	80–300 ng/ml	1.8–6%

[a]Predicted plasma F.IX level in mice based on mouse experimental data[1]. [b]Predicted plasma F.IX level in dogs based on canine experimental data[2]. [c]Extrapolated from studies in animals.

circulation. The data gathered so far indicate that AAV-mediated gene therapy for haemophilia B is safe and has the potential to demonstrate efficacy, although testing at higher doses will be required to confirm this interpretation. This treatment strategy thus offers the possibility of converting severe haemophilia B to a milder form of the disease through a relatively non-invasive procedure. In the broader context of gene-based treatment of inherited diseases, the record so far has been discouraging, with no clear-cut evidence of success with *in vivo* gene therapy. Our results indicate that *in vivo* administration of viral vectors offers the possibility of improving the clinical course of genetic diseases that affect many individuals worldwide.

Methods

Clinical protocol. Our study was designed as an open-label, dose-escalation Phase I trial. The clinical protocol was reviewed and approved by the Institutional Review Boards of The Children's Hospital of Philadelphia (CHOP), Stanford University and the University of Pittsburgh Medical Center, the Institutional Biosafety Committees at CHOP and Stanford (the institutions where vector is injected), the Center for Biologics Evaluation and Research of the U.S. Food and Drug Administration, and the NIH Office for Recombinant DNA Activities. Inclusion criteria for the study include severe haemophilia B with F.IX level ≤1%, life expectancy of at least one year, male sex, age ≥18 years, >20 days exposure history to F.IX concentrates and ability to give informed consent. Exclusion criteria include acute infectious illness, end-stage renal disease, severe liver disease defined as bilirubin >2 times normal, transaminases >5 times normal or alkaline phosphatase >5 times normal, platelet count <50,000, presence of inflammatory muscle disease, unwillingness to practice birth control until three semen samples are documented to be negative for vector sequences and unwillingness to stop a regimen of prophylactic clotting factor infusion. The mutation in *F9* was determined for each patient by the dideoxynucleotide chain termination method following PCR amplification of the eight exons of *F9* from genomic DNA that had been isolated from patient blood samples.

Preparation of AAV-CMV-hF.IX. Vector was prepared in a GMP facility (Avigen) using a triple-transfection procedure[10,11]. The F.IX expression plasmid is an 11,442-bp plasmid containing the cytomegalovirus (CMV) immediate early promoter, exon 1 of *F9* (ref. 12), a 1.4-kb fragment of *F9* intron 1 (ref. 13), exons 2–8 of *F9*, 227 bp of *F9* 3′ UTR and the SV40 late polyadenylation sequence between two AAV inverted terminal repeats. The rep/cap plasmid pHLP19 and the helper adenovirus plasmid pLadeno5 have been described[10,11]. Recombinant AAV was produced by transfecting the three plasmids into HEK 293 cells by calcium phosphate transfection. Following incubation to allow vector amplification, cells were lysed and treated with nuclease to reduce residual cellular and plasmid DNA. After precipitation, vector was purified by two cycles of isopycnic ultracentrifugation; fractions containing vector were pooled, dialysed and concentrated. The concentrated vector was formulated, sterile filtered (0.22 μM) and aseptically filled into glass vials. Vector genomes were titred by a quantitative dot-blot assay in which the signal from aliquots of test material is compared with a standard curve generated using the linearized F.IX expression plasmid. The vector underwent in-process and final product testing as described[14].

Vector administration. After giving informed consent, patients were admitted to the Clinical Research Center at either CHOP or Stanford University for history, physical examination and baseline laboratory studies. On day 0 of the protocol, patients were infused with F.IX concentrate to bring factor levels up to ~100%, and, under ultrasound guidance, vector was injected percutaneously into 10–12 sites in the *vastus lateralis* of either or both anterior thighs. Injectate volume at each site was 250–500 μl, and sites were at least 2 cm apart. Local anaesthesia to the skin was provided by ethyl chloride or eutectic mixture of local anaesthetics (EMLA). To facilitate subsequent muscle biopsy, the skin overlying several injection sites was tattooed and the injection coordinates recorded by ultrasound. We observed patients in the hospital for 24 h after injection; routine isolation precautions were observed during this period to minimize any risk of horizontal transmission of vector. Patients are then discharged and seen daily in outpatient clinic for the next three days, then weekly at the home haemophilia centre for the next eight weeks, twice monthly up to five months, monthly for the remainder of the year, then annually in follow-up. Patients are instructed to infuse factor as usual for haemorrhagic episodes.

Laboratory studies. Laboratory studies drawn in follow-up included F.IX level, aPTT, Bethesda assay, anti-AAV neutralizing antibody titre, routine chemistries, muscle enzymes, CBC, urinalysis, HIV viral load for HIV positive patients, fragment 1.2, and collection of serum, semen, urine, saliva and stool for PCR detection of vector sequences. Patients underwent muscle biopsy of injected sites at 2, 6 and 12 months after injection; studies on skeletal muscle included routine haematoxylin and eosin staining, immunohistochemical staining for F.IX expression, PCR for vector sequences on extracted DNA and Southern blot with a vector probe if adequate amounts of muscle DNA were available. Whenever possible, an effort was made to draw blood samples before factor infusion if a haemorrhagic episode required treatment. All studies were performed in routine clinical laboratories (at CHOP and Stanford) using CLIA-approved procedures, except F.IX ELISA, immunostaining of muscle for F.IX expression, anti-AAV neutralizing antibody titre, PCR of body fluids for vector sequences and western blot to detect anti-F.IX antibodies. We carried out F.IX ELISA as described[15]. For immunohistochemical staining, frozen muscle tissue was cryosectioned and stained using a goat anti-human F.IX antibody (Affinity Biologicals; 1:800 dilution) as described[1], except that a biotinylated horse anti-goat IgG was used as a secondary antibody (1:200 dilution) for immunoperoxidase staining using a kit (Vector Laboratories). Sections were counter-stained with Myers haematoxylin stain.

Antibody assays. We determined AAV neutralizing antibody titres by incubating an AAV vector expressing *lacZ* with serial dilutions of patient serum, then used this cocktail to transduce HEK 293 cells. We lysed cells after 24 h and assayed enzymatic activity using the o-nitrophenyl β-D-galactopyranoside (ONPG) assay[16]. Samples were read at OD_{420} to measure β-galactosidase activity; sera were scored as positive for neutralizing AAV antibodies if the OD_{420} was ≤50% that observed when rAAV-lacZ was pre-incubated with negative control mouse sera. Positive samples were titred; AAV neutralizing antibody titres are presented as dilutions that inhibit infection of rAAV-lacZ by 50% based on the ONPG assay. We carried out western-blot analysis to detect anti-F.IX antibodies. Purified human F.IX was electrophoresed on a 7.5% polyacrylamide gel and transferred to a nitrocellulose membrane using an electroblot system (Biorad). The membrane was incubated with a 1:1,000 dilution of the patient's serum sample as primary antibody and 1:10,000 dilution of anti-human IgG peroxidase conjugate using a chemiluminescent substrate (Pierce) as a detecting antibody.

Viral shedding. We used a PCR assay to detect vector sequences in body fluids (serum, urine, saliva, semen and stool) and biopsied muscle. The 5′ primer (5′–AGTCATCGCTATTACCATGG–3′) was derived from the CMV enhancer and the 3′ primer (5′–GATTTCAAAGTGGTAAGTCC–3′) from

intron 1 of human *F9*. Amplified vector sequence yields a PCR fragment of 743 bp. For each sample, a control reaction containing the sample to be tested spiked with vector plasmid (50 copies/μg DNA) was also run to establish that the sample did not inhibit the PCR reaction. For semen, 3 μg of DNA was analysed (1 μg in each of 3 separate reactions); for saliva and biopsied muscle, 1 μg; and for urine, serum and stool, DNA was extracted from a 1–2 ml volume and analysed. The sensitivity of the assay is 50 copies of vector sequence in 1 μg DNA.

Factor IX levels. We determined F.IX levels using an automated analyser (MDA, Organon-Teknika, or MLA-800, Medical Laboratory Automation). Plasma test samples were mixed with F.IX-deficient substrate (George King, Inc.), and results compared with the degree of correction obtained when dilutions of verify reference plasma were added to the same F.IX-deficient substrate. The reference curve was linear down to a lower limit of 0.3%.

The F.IX measurements reported here deserve comment, as the changes are small. Most clinical laboratories do not report a numerical value for clotting factor levels of <1%, but in preparation for this trial, the coagulation laboratories at CHOP and Stanford University Medical Center prepared detailed standard curves for F.IX, which were linear down to levels of ~0.3%. Most authorities would agree that an experienced clinical coagulation laboratory can distinguish between levels <1% and >1%. The values of >1% in patient A were actually repeated and verified by a third clinical laboratory. Thus it appears that these numbers represent an increase from the patient's true baseline, which was also verified to be <1% by three clinical laboratories before the beginning of the trial.

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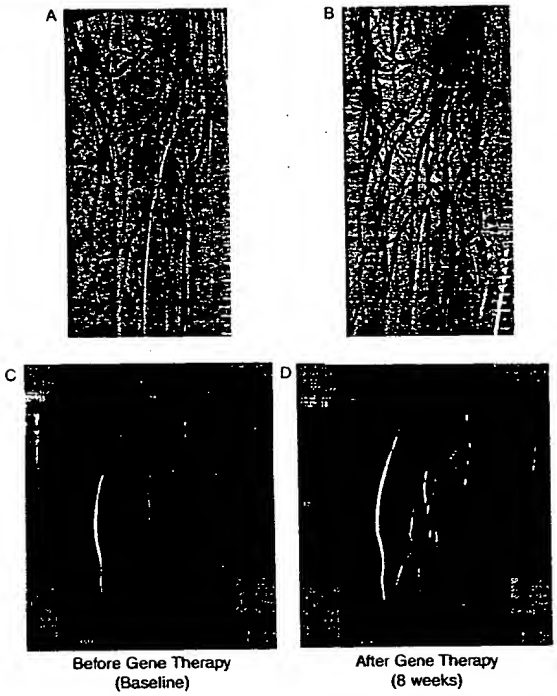

Before Gene Therapy (Baseline)

After Gene Therapy (8 weeks)

Constitutive Expression of phVEGF$_{165}$ After Intramuscular Gene Transfer Promotes Collateral Vessel Development in Patients With Critical Limb Ischemia

Iris Baumgartner, MD; Ann Pieczek, RN; Orit Manor, PhD; Richard Blair, MD; Marianne Kearney, BS; Kenneth Walsh, PhD; Jeffrey M. Isner, MD

Background—Preclinical studies have indicated that angiogenic growth factors can stimulate the development of collateral arteries, a concept called "therapeutic angiogenesis." The objectives of this phase 1 clinical trial were (1) to document the safety and feasibility of intramuscular gene transfer by use of naked plasmid DNA encoding an endothelial cell mitogen and (2) to analyze potential therapeutic benefits in patients with critical limb ischemia.

Methods and Results—Gene transfer was performed in 10 limbs of 9 patients with nonhealing ischemic ulcers (n=7/10) and/or rest pain (n=10/10) due to peripheral arterial disease. A total dose of 4000 μg of naked plasmid DNA encoding the 165-amino-acid isoform of human vascular endothelial growth factor (phVEGF$_{165}$) was injected directly into the muscles of the ischemic limb. Gene expression was documented by a transient increase in serum levels of VEGF monitored by ELISA. The ankle-brachial index improved significantly (0.33±0.05 to 0.48±0.03, P=.02); newly visible collateral blood vessels were directly documented by contrast angiography in 7 limbs; and magnetic resonance angiography showed qualitative evidence of improved distal flow in 8 limbs. Ischemic ulcers healed or markedly improved in 4 of 7 limbs, including successful limb salvage in 3 patients recommended for below-knee amputation. Tissue specimens obtained from an amputee 10 weeks after gene therapy showed foci of proliferating endothelial cells by immunohistochemistry. PCR and Southern blot analyses indicated persistence of small amounts of plasmid DNA. Complications were limited to transient lower-extremity edema in 6 patients, consistent with VEGF enhancement of vascular permeability.

Conclusions—These findings may be cautiously interpreted to indicate that intramuscular injection of naked plasmid DNA achieves constitutive overexpression of VEGF sufficient to induce therapeutic angiogenesis in selected patients with critical limb ischemia. (***Circulation.*** **1998;97:1114-1123.**)

Key Words: angiogenesis ■ genes ■ ischemia ■ growth substances

Critical limb ischemia is estimated to develop in ≈500 to 1000 individuals per million per year.[1] In a large proportion of these patients, the anatomic extent and the distribution of arterial occlusive disease make the patients unsuitable for operative or percutaneous revascularization, and the disease thus frequently follows an inexorable downhill course.[2,3] Psychological testing of such patients has disclosed quality-of-life indices similar to those of patients with cancer in the terminal phase of their illness.[4] As concluded in the Consensus Document of the European Working Group on Critical Limb Ischemia,[1] no pharmacological treatment has been shown to favorably affect the natural history of critical limb ischemia.[5] Indeed, amputation, despite its associated morbidity, mortality, and functional implications,[1,6-8] is often recommended as a solution to the disabling symptoms, in particular excruciating ischemic rest pain, of critical limb ischemia.[9-12] A second major amputation will be required in nearly 10% of such patients. Despite the use of prosthetics and rehabilitation, reestablishment of full mobility is inconsistently achieved, particularly in the elderly. Consequently, the need for alternative treatment strategies in patients with critical limb ischemia is compelling.

See p 1108

Preclinical studies have indicated that angiogenic growth factors can stimulate the development of collateral arteries in animal models of peripheral[13,14] and myocardial[15-17] ischemia, a concept called therapeutic angiogenesis. Several of these studies have used VEGF, also known as vascular permeability factor, a secreted endothelial-cell mitogen with high-affinity binding sites limited to endothelial cells.[18-22] Endothelial cell specificity has been considered to represent an important advantage of VEGF for therapeutic angiogenesis, because endothelial cells represent the critical cellular element responsible for new vessel formation.[23-25]

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Selected Abbreviations and Acronyms

ABI = ankle-brachial index
MRA = magnetic resonance angiography
PCR = polymerase chain reaction
phVEGF$_{165}$ = plasmid encoding 165-amino-acid isoform of human VEGF
TBI = toe-brachial index
VEGF = vascular endothelial growth factor

We recently demonstrated angiographic and histological evidence of angiogenesis after intra-arterial gene transfer of naked plasmid DNA encoding human VEGF in a patient with critical limb ischemia.[26] In this report, we present the results of intramuscular phVEGF$_{165}$ gene transfer performed in an initial phase 1 clinical trial comprising 9 patients with 10 critically ischemic limbs.

Methods

Patients

Patients qualified for intramuscular gene therapy if they (1) had chronic critical limb ischemia[1] including rest pain and/or nonhealing ischemic ulcers present for a minimum of 4 weeks without evidence of improvement in response to conventional therapies and (2) were not optimal candidates for surgical or percutaneous revascularization.[27] Requisite hemodynamic deficit included a resting ABI <0.6 and/or TBI <0.3 in the affected limb on 2 consecutive examinations performed at least 1 week apart. Criteria used to describe a change in limb status were adapted from standards recommended by the Society for Vascular Surgery/North American Chapter and International Society for Cardiovascular Surgery.[3,28] Patients were allowed to continue on aspirin and coumarin, provided that these therapies had been used for a minimum of 6 months before gene transfer. Vasoactive medications were discontinued unless prescribed for cardiac disease or systemic hypertension. All patients gave written informed consent for their participation. The study was designed as a phase 1, nonrandomized study to document the safety of intramuscular phVEGF$_{165}$ gene transfer and to monitor patients as well for evidence of bioactivity. This study design was unanimously approved by the Recombinant DNA Advisory Committee of the National Institutes of Health, by the Human Institutional Review Board and Institutional Biosafety Committee of St Elizabeth's Medical Center, and by the US Food and Drug Administration.

Plasmid DNA (phVEGF$_{165}$)

All patients received a eukaryotic expression vector encoding the VEGF$_{165}$ gene[29] transcriptionally regulated by the cytomegalovirus promoter/enhancer.[26] Preparation and purification of the plasmid from cultures of phVEGF$_{165}$-transformed *Escherichia coli* were performed in the Human Gene Therapy Laboratory at St Elizabeth's Medical Center by the column method (Qiagen Mega Kit, Qiagen, Inc). The purified plasmid was stored in vials and pooled for quality control analyses.

Intramuscular phVEGF$_{165}$ Transfer

Aliquots of 500 μg of VEGF$_{165}$ pDNA were diluted in sterile saline, and 4 aliquots (total, 2000 μg) were administered into calf and/or distal thigh muscles of the patients by direct intramuscular injection into the ischemic limb. The injection sites were arbitrarily selected according to available muscle mass and included sites above as well as below the knee. The volume of each of the 4 injectates per limb was progressively increased during the course of the study from 0.75 mL (3 treatments) to 3 mL (6 treatments) to 5 mL (11 treatments). Four weeks after the first 2000-μg injection, a second 2000-μg injection was administered, increasing the total amount of pDNA to 4000 μg per patient. One patient was treated for bilateral critical limb ischemia with a total amount of 8000 μg pDNA (4000 μg per limb).

Serum VEGF Levels

ELISAs were performed at baseline and weekly up to 12 weeks after the initial treatment of 7 limbs to detect evidence of gene expression at the protein level. Samples were immediately centrifuged for 20 minutes at 3600 rpm at 4°C, and the serum was stored at −20°C until analysis. Serum VEGF was determined with an immunoassay according to the manufacturer's instructions (R&D Systems). Results were compared with a standard curve of human VEGF with a lower detection limit of 5 pg/mL. Samples were checked by serial dilution and were performed at least in duplicate.

Hemodynamic and Angiographic Assessment

Patients were followed up on a weekly basis within the first 8 weeks after gene therapy and at monthly intervals thereafter. Ischemic ulcers were documented by color photography. Resting ABI and TBI were calculated by the quotient of absolute ankle or toe pressure to brachial pressure.[30] Intra-arterial digital subtraction angiography and MRA were performed within 1 week before and 4 weeks after each treatment and 3 months after the latter of 2 intramuscular injections. Digital subtraction angiography was performed as a selective single-leg runoff study using undiluted nonionic contrast media (Isovue-370, Squibb Diagnostics). A minimum of 2 images (early and late frames) at the thigh, knee, calf, and ankle/foot levels were recorded by digital acquisition and hard copies in a 35×45-cm format. The diameter of newly visible collateral vessels was assessed by comparison with a 0.09-in-diameter reference wire taped to the skin. MRA was performed with a 1.0-T superconducting system (Impact, Siemens) by means of a transmit-receive extremity coil, a body coil, or both and commercially available pulse sequences. A multisection two-dimensional time-of-flight gradient echo sequence without intravenous contrast medium was used.[31] All axial images were reconstructed by use of the maximum-pixel-intensity algorithm at intervals of 60°.

Immunohistochemistry

Double immunohistochemical staining for proliferating endothelial cells was performed as previously described.[32] Bound antibody was then detected with an alkaline phosphatase kit (Biogenex Laboratories). Complexes were visualized with fast red substrate (Biogenex Laboratories). A counterstain of 10% Gill hematoxylin was applied before coverslips were applied.

DNA Analysis

Skin specimens at the site of gene injection and muscle specimens near or remote from the site of gene injection were retrieved from 2 amputees 8 and 10 weeks after intramuscular phVEGF$_{165}$ transfer, respectively (patients 4 and 10, Table). Tissue was processed with a genomic DNA isolation kit (A.S.A.P., Boehringer Mannheim). For PCR analysis, primer sets unique to the promoter and VEGF coding region of phVEGF$_{165}$ were selected. For Southern analysis, *Eco*R1-digested total cellular DNA (30 μg) and purified phVEGF$_{165}$ DNA (0.5 μg) were subjected to 0.8% agarose electrophoresis. The predicted sizes of *Eco*R1-digested plasmid fragments were 998 and 4703 bp. DNA blotted to a nylon membrane (Amersham, Life Science) was hybridized with two ^{32}P-labeled phVEGF$_{165}$-specific probes (*nco*I-digested 679-bp phVEGF$_{165}$ fragment, position 389 to 1068; *ava*I-digested 787-bp phVEGF$_{165}$ fragment, position 991 to 1778), washed, and exposed to Hyperfilm MP (Amersham, Life Science).

Statistical Analysis

Data are reported as mean±SEM. Comparisons between paired variables were performed with the nonparametric Friedman test and Wilcoxon rank sum test. All statistical tests were two-tailed, with a significance level of $P<.05$.

Clinical, Hemodynamic, Angiographic, Laboratory, and Molecular Findings Before and After Intramuscular phVEGF$_{165}$ Gene Transfer

			Clinical History and Findings Before Gene Therapy				Outcome After Gene Transfer	
No.	Sex	Age, y	Cigs, pk/y	DM	Previous Treatment	Signs/Symptoms	Limb Status	DSA Findings
1	F	33†	30	0	4 bypass grafts, 3 rev., prostaglandins	Calf ulcer, toe gangrene (digit I)	ABI +0.24; complete healing→limb salvage	New collaterals, 200–400 μm
2	F	53	0	+	3 bypass-grafts, 1 PTA, prostaglandins	Toe gangrene (digit V)	ABI +0.12; complete healing	New collaterals, 200–400 μm
3	M	77	0	+	None	Toe gangrene (digits I, IV)	TBI +0.11; gangrene/ osteomyelitis→BKA	New collaterals, 200–400 μm
4	F	39†	20	0	Sympathectomy	Forefoot gangrene	ABI +0.27; forefoot necrosis→BKA	New collaterals, 200–400 μm
5	M	74	90	0	1 PTA	Rest pain	ABI +0.15; rest pain resolved	New collaterals, 200–800 μm
6	F	84	40	0	6 bypass grafts, 1 PTA	Toe gangrene (digits I–V)	ABI +0.22; toe amputation→limb salvage	None
7	F	80	20	0	1 bypass graft	Rest pain	ABI unchanged, rest pain resolved	New collaterals, 200–800 μm
8*	F	39	20	0	Sympathectomy	Heel ulcer, toe gangrene (digits I–IV)	ABI +0.22; toe amputation→limb salvage	New collaterals, 200–>800 μm
9	M	54	30	0	4 bypass grafts, 2 rev., 1 PTA	Rest pain	TBI +0.18; rest pain resolved	None
10	M	54	70	0	6 bypass-grafts, 1 PTA	Toe gangrene (digits I, III, IV)	No change in ABI/TBI, BKA	None

No. indicates consecutively treated ischemic limbs; Cigs, current cigarette smoker; pk/y, pack years of cigarette smoking; DM, diabetes mellitus (non–insulin-dependent DM, oral medication); DSA, digital subtraction angiography; BI, baseline; rev., surgical revisions; PTA, percutaneous transluminal angioplasty; TBI (ABI incompressible); BKA, below-knee amputation; ND, not done; and pos., positive.

*No. 8 and 4 identical patient (bilateral treatment).

†Suspected Buerger's disease (stopped smoking >3 months before study entry).

Results

Demographic and clinical data for the 5 women and 4 men (mean age, 59±19 years) treated with phVEGF$_{165}$ are shown in the Table. Average length of follow-up at the time of this report was 6±3 months (range, 2 to 11 months). Local intramuscular gene transfer induced no or mild local discomfort up to 72 hours after the injection. Serial creatine phosphokinase measurements remained in the normal range, there were no signs of systemic or local inflammatory reactions, and no patient developed antibodies to VEGF. To date, neither loss of visual acuity nor change in funduscopic examination due to diabetic retinopathy[33] has been observed in any patient treated with phVEGF$_{165}$ gene transfer. Likewise, no development of a latent neoplasm[34] has been observed. The only complication seen was transient lower-extremity edema, consistent with VEGF enhancement of vascular permeability.[35]

Transgene Expression

Blood levels of VEGF transiently peaked 1 to 3 weeks after gene transfer in 7 patients in whom weekly blood samples were obtained (Fig 1). (In 2 patients, baseline and/or more than two follow-up blood samples were not obtained.) Indirect clinical evidence of VEGF overexpression was evident from the development of peripheral edema (+1 to +4 by gross inspection) in the 6 patients with ischemic ulcers. In 4 of these patients, edema was limited to the treated limb, whereas in 2 patients, the contralateral limb was affected as well, albeit less severely. The onset of edema corresponded temporally to the rise in serum VEGF levels.

Noninvasive Arterial Testing

Absolute systolic ankle or toe pressure increased in 8 limbs after gene transfer and was unchanged in 1 limb at the time of the most recent follow-up (53±4.8 at baseline, 66±4.6 most recent follow-up, P=.008). ABI and/or TBI increased from 0.33±0.05 (range, 0 to 0.58; n=10) at baseline to 0.43±0.04 (0.22 to 0.57, P=.028; n=10) at 4 weeks, to 0.45±0.04 (0.27 to 0.59, P=.016; n=10) at 8 weeks, and to 0.48±0.03 (0.27 to 0.67, P=.017; n=8) at 12 weeks (Fig 2). Improvement in the pressure index was sustained, but the increases in values obtained after the second (4-week) injection were not significantly different from measurements made 4 weeks after the initial injection. Exercise performance improved in all 5 patients with rest pain or ischemic ulcers who were able to perform graded treadmill exercise.[36] All patients experienced a significant increase in pain-free walking time (2.5±1.1 minutes before gene therapy versus 3.8±1.5 minutes at an average of 13 weeks after gene therapy, P=.043). A statistically significant increase in absolute, claudication-limited

Continued

VEGF Level, pg/mL			
BI	1st	2nd	Molecular Findings
47	223	607	
36	ND	ND	
46	131	780	
30	59	888	PCR pos. in skin + muscle specimens; Southern pos. in muscle specimens
62	300	96	
29	164	80	
40	44	223	
ND	ND	ND	
0	113	83	
ND	ND	ND	PCR pos. in skin + muscle specimens; Southern pos. in muscle specimens

walking time (4.2±2.1 minutes before versus 6.7±2.9 minutes after gene therapy, $P=.018$) was documented as well. Two patients reached the target end point of 10 minutes of exercise.

Angiography

Digital subtraction angiography showed newly visible collateral vessels at the knee, calf, and ankle levels in 7 of 10 treated ischemic limbs. The luminal diameter of the newly visible vessels ranged from 200 to >800 μm, although most were closer to 200 μm; the latter frequently appeared as a "blush" of innumerable collaterals (Fig 3A and 3B). Follow-up angiograms disclosed no evidence of collateral artery regression in any patients. Serial magnetic resonance angiograms of the ischemic limb disclosed qualitative evidence of improved distal blood flow in 8 limbs, including enhancement of signal intensity in previously identified vessels, and an increase in the number of newly visible collaterals (Fig 3C and 3D).

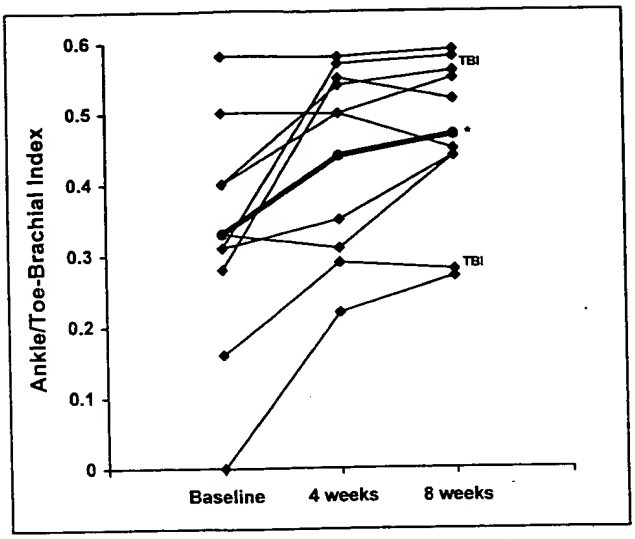

Figure 2. Gain in ABI and/or TBI in 10 limbs 4 and 8 weeks after intramuscular phVEGF$_{165}$ gene transfer. *Mean values, $P=.02$.

Change in Limb Status and Ischemic Rest Pain

Therapeutic benefit was demonstrated by regression of rest pain and/or improved tissue integrity in the ischemic limb. Limb salvage, for example, was achieved in a 33-year-old woman (patient 1, Table), who had undergone 7 unsuccessful surgical reconstructions at another hospital. She presented with a necrotic great toe and a 9×3-cm ischemic ulcer at the site of vein harvest in her distal left limb (Fig 4). The ulcer had failed to respond to 6 months of conservative measures, during the last 3 of which she had been treated with methadone, oxycodone/acetaminophen, amitriptyline hydrochloride, and a fentanyl patch. She had been advised by her vascular surgeons to undergo below-knee amputation. Within 8 weeks after gene transfer, her ABI had increased by 0.24, and the ulcer dimensions had diminished sufficiently to permit placement of a split-thickness skin graft. The graft healed successfully and remained healed at 9-month fol-

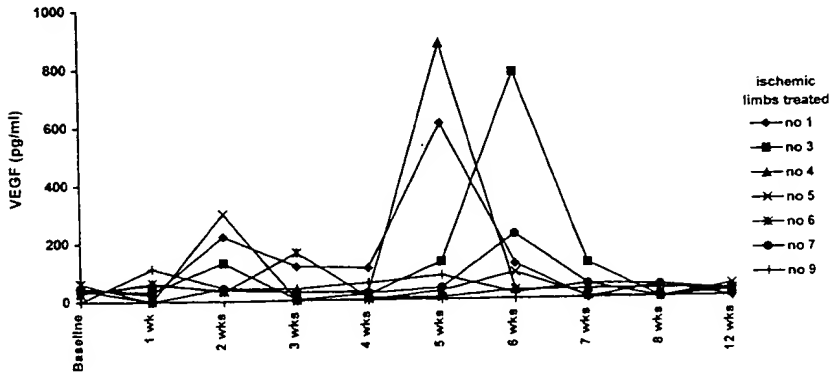

Figure 1. Serial levels of VEGF determined by ELISA disclosed a transient elevation 1 to 2 weeks after intramuscular (phVEGF$_{165}$) gene transfer. Baseline and/or weekly follow-up venous blood samples, which were incomplete in 3 of 10 treated limbs (patients 2, 8, and 10 in the Table), are not shown.

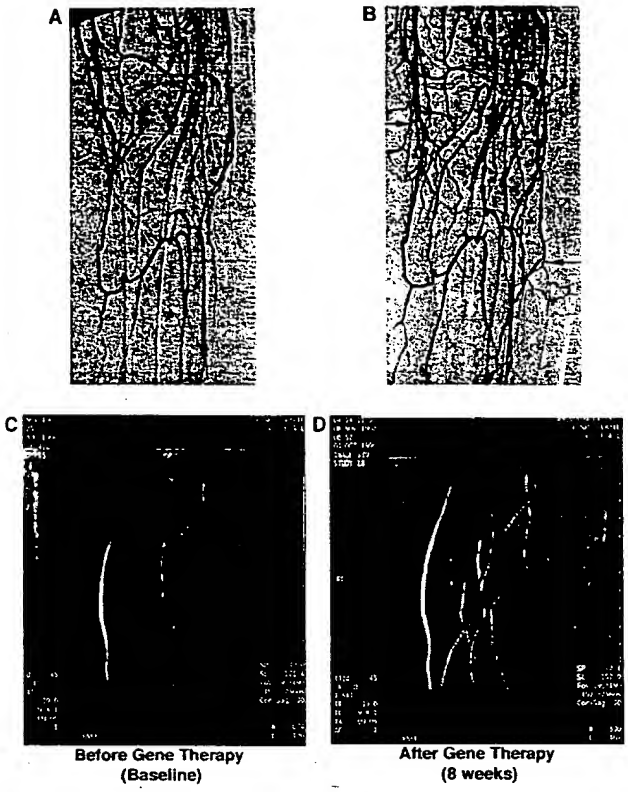

Figure 3. A and B, Newly visible collateral vessels at calf level 8 weeks after phVEGF$_{165}$ gene transfer. Luminal diameter of newly visible vessels ranged from 200 to >800 μm (arrow); most were closer to 200 μm, and these frequently appeared as a blush of innumerable collaterals. C and D, MRA before and 8 weeks after gene therapy. After gene therapy, signal enhancement is clearly evident, consistent with improved flow in ischemic limb.

low-up (Fig 4). A second patient, a 39-year-old woman (patients 4 and 8, Table), presented with a 3-month history of gangrene of the distal half of her right foot. Although the ABI in her right limb improved by 0.27 after gene transfer, the forefoot gangrene was not reversed, and she underwent right below-knee amputation. While she was being treated for the right limb, however, she developed gangrene in the left limb (Fig 5). After gene transfer to the left limb, the ABI in her left lower extremity increased by 0.22 in association with angiographically demonstrable, newly visible collateral vessels. Although she required amputation of the left great toe, the operative site healed promptly, and her remaining 4 toes and heel recovered completely, including restoration of normal nail growth (Fig 5). In 2 other patients, complete (patient 2, Table) or partial (patient 6, Table) healing of ischemic ulcers present for 12 and 2 months, respectively, avoided major limb amputation. In the 3 patients (patients 5, 7, and 9, Table) who presented with rest pain (of 6, 5, and 3 months' duration, respectively) unassociated with loss of tissue integrity, rest pain resolved completely in all 3 patients after gene transfer and has remained so at the most recent follow-up.

For the total group of 10 limbs, frequency of ischemic rest pain expressed as afflicted nights per week decreased significantly (5.9±2.1 at baseline versus 1.5±2.8 at 8-week follow-up, P=.043). On the basis of criteria proposed by Rutherford et al,[3] limb status improved in 9 of 10 extremities treated (Table). Moderate improvement, including both an upward shift in clinical category (≥1 clinical category in patients with rest pain and ≥2 categories in patients with tissue loss) and an increase of >0.1 in the ABI, was documented in 5 patients.

Immunohistochemistry and Molecular Analyses

Tissue specimens retrieved from 1 amputee 10 weeks after gene therapy showed foci of proliferating endothelial cells (Fig 6A). This finding was particularly striking given that endothelial cell proliferation is nearly absent in normal arteries.[37] PCR performed on these samples indicated persistence and anatomic redistribution of DNA fragments unique to phVEGF$_{165}$. Noteworthy amplification of DNA fragments was shown in muscle and skin samples derived from the site of injection as well as in several muscle samples remote from the injection site (Fig 6B). Southern blot analysis confirmed persistence of intact plasmid DNA in muscle specimens derived from 2 amputees 8 and 10 weeks after gene therapy (patients 4 and 10, Table) (Fig 6C).

Discussion

The natural history of critical limb ischemia has been well documented to have an inexorable downhill course.[3] The inclusion criteria for this study were drafted to restrict treatment to patients in whom the natural history of critical limb ischemia had been established previously. Seven of the 10 limbs had developed frank gangrene. Although inclusion criteria required a minimum of 4 weeks of conservative measures without evidence of improvement, in reality, signs and/or symptoms of critical limb ischemia had been present in all cases for 2 to 12 months before gene therapy. Among this series of 9 patients (10 limbs), 6 developed critical limb ischemia despite having undergone as many as 7 vascular surgical reconstructions. Seven patients had been specifically advised to undergo limb amputation. All were using analgesic, typically ≥1 narcotic, medications. Spontaneous resolution of rest pain and/or healing of an ischemic ulcer in patients like these with critical limb ischemia has not to our knowledge been reported previously.[1] Furthermore, because VEGF had not been administered previously as recombinant protein, no data were available from any source to indicate either the safety or bioactivity of any dose of phVEGF$_{165}$. Accordingly, the design of this phase 1 trial, unanimously approved by the Recombinant DNA Advisory Committee and the US Food and Drug Administration, was conducted as a nonrandomized, consecutive treatment series, similar to phase 1 oncology protocols used to study new chemotherapeutic agents administered to human subjects.

Analysis of gene expression at the protein level by use of an ELISA assay for VEGF documented a transient peak of the gene product in the systemic circulation 1 to 3 weeks after gene transfer in 7 patients. Further evidence of gene expression was observed in 6 patients, who developed temporally related peripheral edema, including 2 with bilateral edema. Parenthetically, the latter finding constitutes what is to the best of our knowledge the first demonstration that VEGF may augment vascular permeability in human subjects.

In most patients, treatment was sufficient to achieve clinically significant modulation of the recipient phenotype.

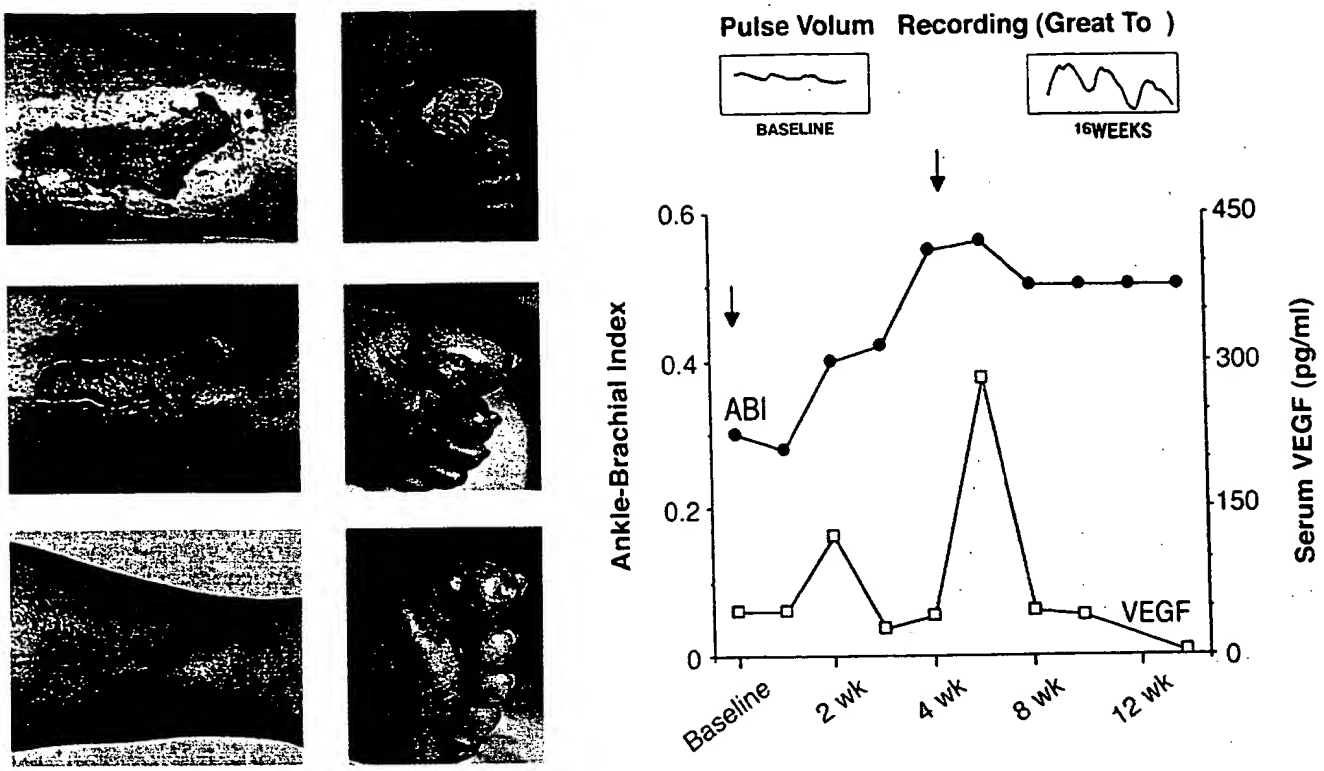

Figure 4. Limb salvage after gene therapy in a 33-year-old woman (patient 1, Table). Left top, Nonhealing wound on medial aspect of calf and ischemic necrosis involving great toe. Left middle, Ingrowth of granulation tissue in calf wound, healing of great toe. Left bottom, Three months after gene transfer, healing of split-thickness skin graft at wound site and full resolution of great toe necrosis. Before gene therapy, patient was wheelchair-bound on multiple analgesics, including methadone, amitriptyline hydrochloride, clonidine, oxycodone/acetaminophen, and a fentanyl patch. Three months after gene transfer, she was freely ambulatory and had been successfully weaned from all analgesics. Right, Evidence of phVEGF$_{165}$ bioavailability documented by an increase in venous VEGF blood levels and bioactivity expressed as an increase in ABI. The ABI progressively increased from 0.28 before to 0.56 after gene therapy (weeks refer to time after transfection). This was associated with development of a phasic pulse volume recording compared with nonphasic tracing recorded at baseline. Vertical arrows indicate timing of each of the 2 intramuscular phVEGF$_{165}$ injections.

Noninvasive studies documented hemodynamic evidence of improved limb perfusion that satisfies outcome criteria proposed to assess the results of surgical reconstruction or percutaneous revascularization.[28] Absolute ankle and/or toe pressure increased in 8 limbs after gene therapy (P=.008). ABI and/or TBI increased from 0.33±0.05 at baseline to 0.48±0.03 at 12 weeks (P=.017). To put this in perspective, an increase of >0.1 in the ABI is considered indicative of a successful surgical or percutaneous intervention.[28] To the best of our knowledge, such improvement has not previously been achieved spontaneously or with medical therapy in patients with critical limb ischemia.

Similarly, angiographic demonstration of newly visible collateral vessels, accompanied here by noninvasive (MRA) evidence of improved blood flow, has to the best of our knowledge not been reported previously in response to any therapeutic intervention. Indeed, previous reports have indicated that current methods used to perform diagnostic contrast angiography cannot provide images of arteries measuring <200 μm in diameter[38]; the spatial resolution of images obtained by MRA is even less. Using synchrotron radiation microangiography to assess collateral artery development after VEGF gene transfer in a rat model of hindlimb ischemia, Takeshita et al[38] showed that neovascularization included a substantial contribution of vessels <180 μm in diameter. Thus, conventional angiographic techniques used in the present study may have failed to depict the full extent of angiogenesis achieved after phVEGF$_{165}$ transfection, particularly given that most newly visible collaterals were diminutive (200 to 800 μm).

That angiogenesis was in fact therapeutic in the present investigation was shown by concomitant reduction in rest pain and/or a favorable impact on limb integrity. Rest pain resolved in all 3 of the patients who presented with rest pain alone. Ischemic ulcers present in 7 limbs healed or improved markedly in 4 patients; this included 3 patients recommended for below-knee amputation in whom successful limb salvage was achieved. Given the poor prognosis for patients with chronic critical limb ischemia, in whom the possibility of spontaneous improvement is remote,[1,2] the outcome in this initial cohort is thus encouraging.

Beginning with the reports of Wolff et al,[39] work from several laboratories[40–48] convincingly demonstrated evidence of transgene expression after direct injection of nonviral, covalently closed pDNA into skeletal muscle. The conceptual basis for therapeutic angiogenesis after phVEGF$_{165}$ gene transfer in particular has been established previously by our laboratory.[49,50] The results of the present trial extend previous findings from studies performed in live animals[46] to patients with advanced peripheral artery disease.

The failure of previous gene therapy trials to yield evidence of clinical success has been attributed to gene delivery,

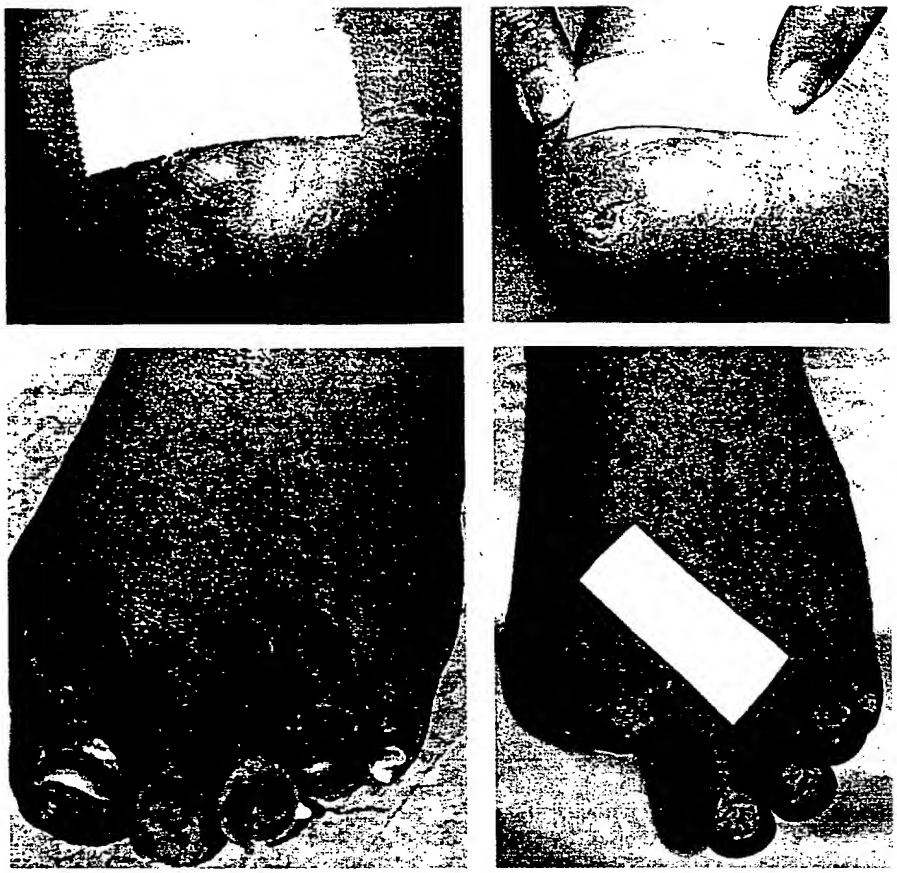

Figure 5. Limb salvage after gene therapy in a 39-year-old woman (patient 8 and 4, Table). This patient presented with a 3-month history of gangrene of distal one-half of right foot. Although ABI in right limb improved by 0.27, forefoot gangrene was not reversed, and she underwent below-knee amputation. While she was being treated for right limb, however, she developed gangrene of left great toe and shortly after, of 4 remaining left toes as well. After gene transfer to left limb, her ABI increased by 0.22 in association with angiographically demonstrable new collateral vessels. Although she required a great toe amputation, operative site healed promptly, and remaining 4 toes recovered completely, including restoration of normal nail growth.

specifically the inability to deliver genes efficiently and to obtain sustained expression.[51] Those cases in which phVEGF$_{165}$ gene therapy led to successful clinical outcomes in this clinical trial suggest that the success of gene therapy is not solely a function of transfection efficiency, nor is it necessarily dependent on protracted gene expression. Several aspects of the gene, protein, and target tissue may have contributed to successful modulation of the host phenotype, despite the relatively low transfection efficiency typically associated with naked DNA. First, VEGF, as noted above, is actively secreted by intact cells; previous studies in our laboratory[52] have documented that genes that encode for secreted proteins, as opposed to proteins that remain intracellular, may yield meaningful biological outcomes because of paracrine effects of the secreted gene product. Second, heparin avidity of the VEGF$_{165}$ isoform promotes binding to cell surface and matrix heparan sulfates that may create a biological reservoir of the secreted protein, enhancing the temporal opportunity for bioactivity. Third, although endothelial cells were previously viewed solely as the target for VEGF, it is now clear that endothelial cells subjected to hypoxia can synthesize VEGF as well.[53] This autocrine feature of VEGF creates the opportunity for amplifying the effects of even a small amount of exogenous VEGF, because endothelial cell proliferation in the ischemic territory creates additional potential cellular sources of VEGF synthesis and secretion. Fourth, VEGF inhibits apoptosis,[54] in part by upregulating endothelial cell expression of fibronectin and $\alpha_v\beta_3$,[54,55] thus preserving the survival signal generated by attachment of endothelial cells to their extracellular matrix. Such reduction in endothelial cell apoptosis would be expected to complement the mitogenic effect of VEGF, resulting in a further net increase in endothelial cell viability. Fifth, with regard to the target of gene therapy, it has been noted[14,26,49] that VEGF-induced angiogenesis is not indiscriminate or widespread but rather is restricted to sites of ischemia. This appears to result from paracrine upregulation of the principal high-affinity VEGF receptor (*Kdr*) in response to factors released from hypoxic skeletal myocytes.[56] Receptor upregulation on endothelial cells within the region of lower-limb or myocardial ischemia thus enables these cells to act as magnets for any VEGF secreted into the ischemic milieu. Finally, the fact that the host tissues are by definition hypoxic may directly aid intramuscular transfer of naked DNA, because transfection efficiency is augmented when the injected skeletal muscle is ischemic.[40,46]

Previous work from our laboratory established that phVEGF$_{165}$ transgene expression is limited to <30 days in animal models of limb ischemia.[26,46,49] Although Southern blot and PCR analyses indicated that small amounts of plasmid DNA were preserved in tissue specimens derived from 2 amputees in this clinical trial, we have no evidence to suggest that transgene expression is more protracted in human subjects than in our animal models. Fortuitously, however, it appears that in both animals and humans, collateral vessel development sufficient to restore limb perfusion to satisfactory resting levels occurs within this time interval. Cessation of gene expression beyond this time point can be considered to constitute an inherent safety feature of phVEGF$_{165}$ gene

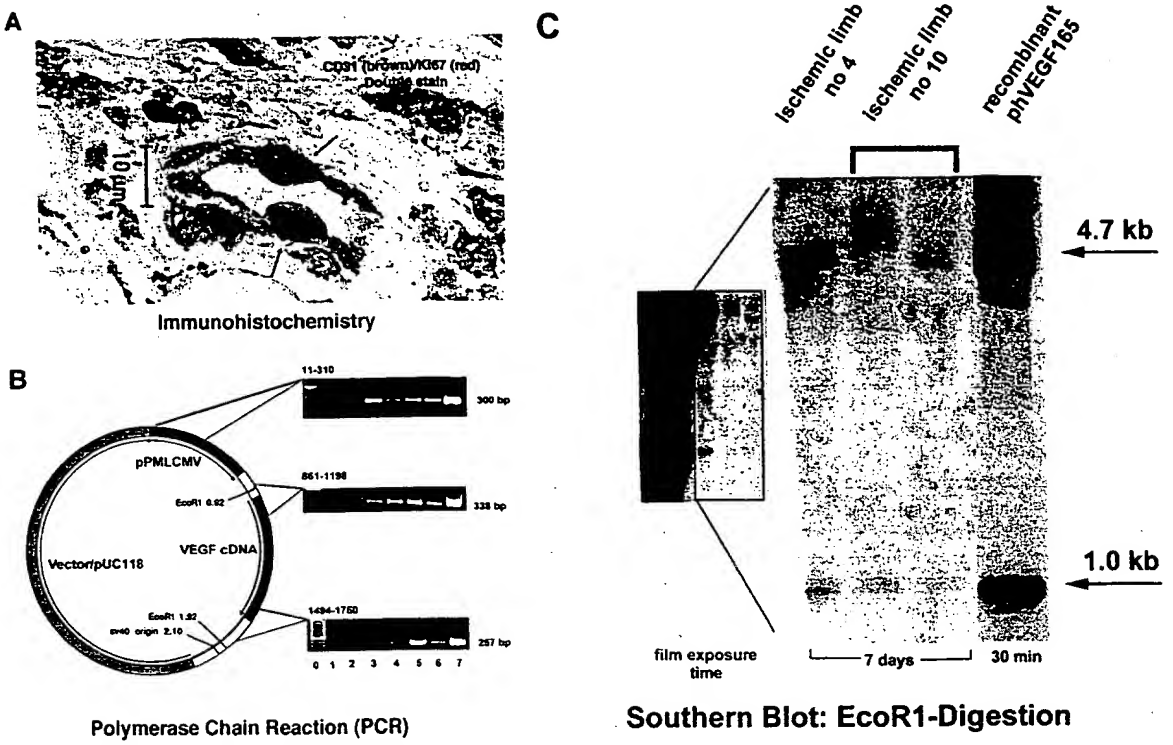

Figure 6. Immunohistochemical and molecular analyses of tissue specimens derived from 2 amputees 8 and 10 weeks after gene therapy (limbs 4 and 10, Table). A, Double immunohistochemical staining of tissue specimen with monoclonal antibody to CD31 (brown) and polyclonal antibody to Ki-67 (red) shows proliferating microvascular endothelial cells (arrows). B, PCR demonstrates amplification of $phVEGF_{165}$ DNA fragments in skin and skeletal muscle specimens. Lane 0 shows 100-bp ladder; lane 2, reaction mixture without tissue DNA; lane 3, negative control (skin specimen from untreated patient); lanes 4 to 6, specimens derived from $phVEGF_{165}$-treated amputee (patient 4, Table); lane 4, skin; lane 5, gastrocnemius muscle (remote from site of transfection); lane 6, tibialis anterior muscle (transfection site); lane 7, positive control (purified $phVEGF_{165}$). Amplified fragments had predicted sizes of 300, 338, and 257 bp, spanning the CMV promoter/enhancer region (5′-CCCGACATTGATTATTGA-3′ and 5′-CGGGCCATTTACCGTCAT-3′; position 11 to 300), proximal VEGF encoding region and junction between VEGF encoding region and promoter (5′-GCCTTTCTCTCCACAGGT-3′ and 5′-GTACTCGATCTCATCAGG-3′; position 861 to 1198), and distal VEGF encoding region and junction between VEGF encoding region and SV40 polyadenylation sequence (5′-CGCGTTGCAAGGCGAGGC-3′ and 5′-GGACCCAAAGTGCTCTGC-3′; position 1494 to 1750), respectively. C, Southern blot analysis of *Eco*R1-digested total cellular DNA (30 µg) (lane 1, patient 4, Table; lanes 2 and 3, patient 10, Table), and 0.5 µg (lane 4) of purified $phVEGF_{165}$ DNA hybridized with two ^{32}P-labeled $phVEGF_{165}$ specific probes (position 389 to 1068 and position 991 to 1778) showed persistence of complete plasmid DNA (*Eco*R1-digested 4703- and 998-bp fragments) in skeletal muscle specimens derived from 2 amputees (patients 4 and 10, Table).

transfer that protects the organism from indefinite constitutive expression of an angiogenic growth factor.

Several caveats regarding this preliminary clinical experience must be acknowledged. First, it is theoretically possible that VEGF expression resulting from gene transfer could promote the development of a tumor that is currently too small to be recognized. Previous laboratory studies, however, have established that VEGF expression, although sufficient to promote a growth process, did not lead to malignant proliferation or to metastasis, a finding in agreement with the notion that stimulation of angiogenesis is necessary but not sufficient for malignant growth.[34,57] It is also theoretically possible that VEGF may aggravate deteriorating eyesight due to diabetic retinopathy.[33] To date, however, no change in visual acuity has been observed in any patient treated with $phVEGF_{165}$ gene transfer. Nevertheless, these findings are preliminary and do not establish the long-term safety of VEGF, administered either as a gene or gene product. Second, although it is conceivable that continuous, predominantly local production of VEGF resulting from the transgene may be preferable, from the standpoints of both safety and efficacy, to a single larger dose of recombinant protein, this notion remains to be proven. Preliminary clinical trials of recombinant VEGF protein therapy have confirmed that mild hypotension seen in preclinical studies[15,58] may be seen in humans as well (unpublished data). Presumably, the route and/or dose of recombinant protein delivery can be adjusted to address this issue. Clearly, further clinical studies of both recombinant protein and alternative dosing regimens of gene therapy will be required to define the relative merits of each approach. Third, we cannot exclude the possibility that these encouraging preliminary results might have been made more substantial and/or uniform by the use of alternative vector systems and/or dosing strategies.[45,48,51,59]

In summary, these preliminary data may be cautiously interpreted to support both the strategy of intramuscular gene therapy and the concept of therapeutic angiogenesis for treatment of selected patients with critical limb ischemia.

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